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13. ABSTRACT (Maximum 200 Words) To improve the efficacy of intravenous administration of herpes vectors for metastatic prostate cancer we conducted experiments in the Tramp model system using clinically applicable agents that have been previously shown to transiently inactivate complement activity and demonstrate that dextran sulfate was effective in enhancing the tumor inhibitory activity of G207 efficiently infects human endothelial cells (HUVEC) at a low MOI 0.01 and kills >80% if cells by day 3. To explore if HSV therapy may beneficially interact with commonly-used chemotherapeutics, G207 was tested in combination with cyclophosphamide (CyP); CyP+G207 was more effective in inhibiting TRAMP-C2 tumor growth compared to either agen alone (p<0.05). In a bilateral TRAMP-C2 model, NV1042, derived from NV1023, with an additional insertion of the cytokine gene, mIL-12, improved tumor regression not only in the treated tumor also in the non-treated contralateral tumors. This immune effect on distant tumors was also observed with the second mouse prostate tumor model, Pr14-2. Further, using systemic administration, a combination of vectors expressing two cytokines NV1042 (IL-12) and NV1034 (GM-CSF) was more effective than either alone.				
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## ANNUAL PROGRESS REPORT FOR AWARD DAMD17-98-1-8490

ROBERT L. MARTUZA, M.D. "Herpes virus therapy of prostate cancer"

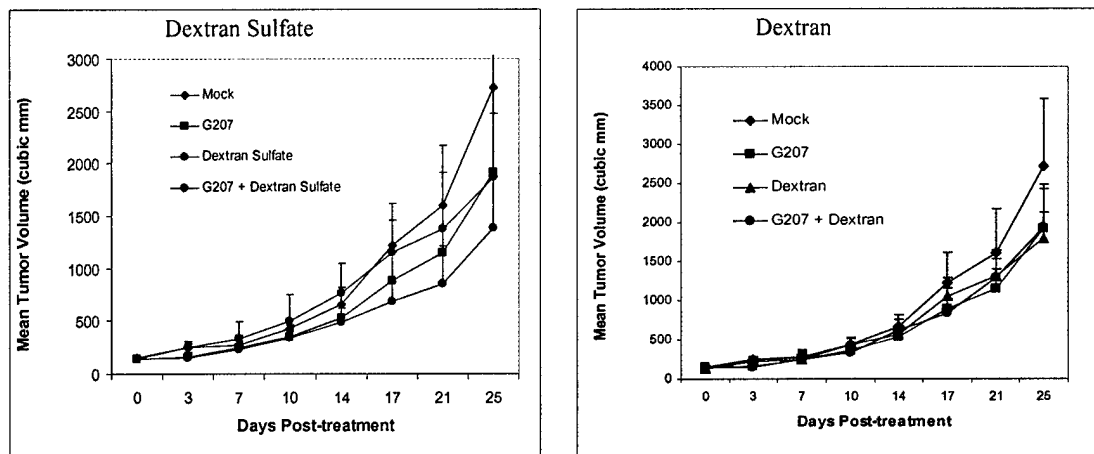
### INTRODUCTION:

Our goals remain the same as those outlined in the original grant proposal where we stated that we expect these studies to lead to improved vector design for local prostate cancer therapy and for systemic therapy for metastatic prostate cancer using oncolytic herpes vectors. We have made significant progress in several of the areas detailed in our original hypotheses and statement of work.

### BODY:

**HYPOTHESIS 1. The efficacy of intravenous administration of herpes vectors for metastatic prostate cancer can be improved with the use of other HSV vectors and part of the efficacy may be due to selective injury of tumor vasculature.**

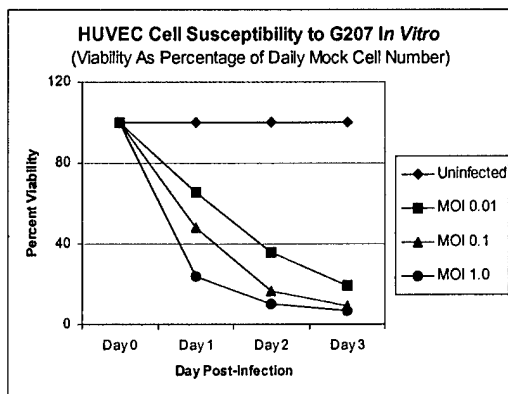
In our previous report we demonstrated that four intravenous administrations of  $2 \times 10^7$  pfu of G207 inhibited TRAMP-C2 tumor growth by 50% of untreated tumor volume. Effectiveness of systemically administered virus is dependent upon several factors, including the dose of the virus and inactivation of the virus particles by complement. The response of TRAMP-C2 tumors to G207 could potentially be improved by blocking complement-inactivation of virus in the immunocompetent C57Bl/6 mice. In order to investigate this, we have conducted experiments in the Tramp model system using clinically applicable agents that have been previously shown to transiently inactivate complement activity, such as dextran sulfate and dextran (Fig. 1). TRAMP-C2 tumors established in C57Bl/6 mice were treated with 2 doses of  $2 \times 10^7$  pfu of G207 given intravenously (tail vein) on days 0 and 3 singly or mixed with



**Fig. 1: Efficacy of Dextran sulfate or Dextran in combination with systemic G207 on mouse prostate tumor growth.** Subcutaneous TRAMP-C2 tumors established in C57Bl/6 mice were treated with  $2 \times 10^7$  pfu of G207 delivered intravenously by tail vein on days 0 and 3 either singly or mixed with 100 $\mu$ g/ml dextran sulfate or dextran.

100 $\mu$ g/ml of either agent. The results demonstrate that dextran sulfate was effective in enhancing the tumor inhibitory activity of G207 ( $p < 0.05$ ). Interestingly, dextran sulfate administration alone also caused some inhibition of tumor growth, although the combination was more effective ( $p < 0.05$ ).

We have previously published that G207 has two mechanisms of tumor cell killing: (1) direct viral toxicity to tumor cells, or "oncolysis", and, (2) an "*in situ* vaccine" effect, in which G207 infection and destruction of tumor cells causes the activation and recruitment of tumor specific cytotoxic T cells to kill not only the infected tumor cells but also non-infected tumor cells at a distance. Additionally, we have hypothesized that a third potential tumoricidal effect of G207 could be on endothelial cells lining the blood vessels that feed the tumor. Because the endothelium of neovasculature is actively proliferating versus the relatively quiescent endothelium of the normal organ, it should be selectively susceptible to this approach. Therefore, *in vitro* studies were conducted to investigate this possibility and these demonstrate that G207 can efficiently infect human endothelial cells (HUVEC) even at a very low MOI of 0.01 and kill more than 80% of cells by day 3 (Fig. 2).



**Fig. 2: Susceptibility of HUVEC to G207 *in vitro*.** HUVEC cells were plated in triplicate wells and infected 24 hours later with varying MOI of G207. Cell viability was determined on day 1-3 following infection. Data is represented as percent of uninfected control cell number for each day.

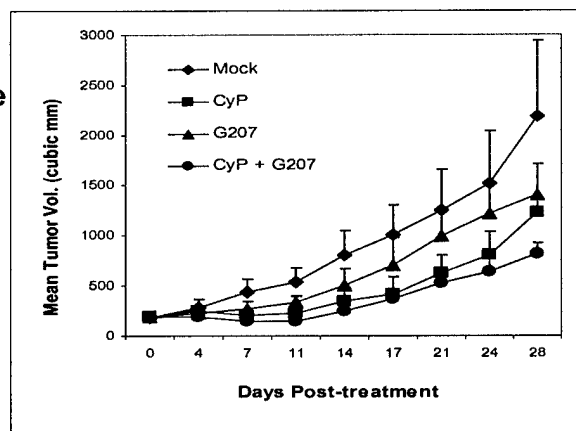
We are now planning studies to confirm this cytolytic effect of G207 on endothelial cells *in vivo* using mouse tumor models.

## **HYPOTHESIS 2. HSV therapy may beneficially interact with commonly-used partially-effective chemotherapy agents.**

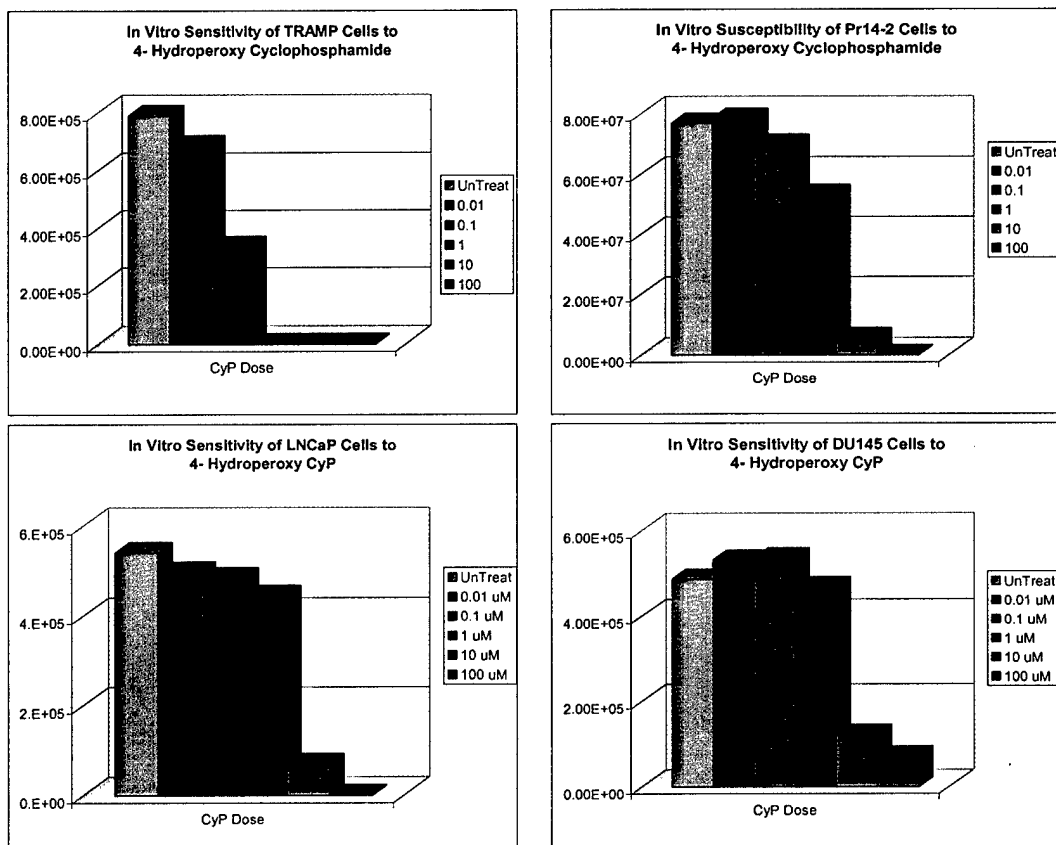
Widely spread hormone-refractory prostate cancer is often treated with chemotherapeutic agents but, due to its partial response, this remains as a palliative measure. In order to improve upon this partial effect of chemotherapy and because its mode of action is distinct from virus therapy, experiments were conducted to test the efficacy of G207 in combination with cyclophosphamide (CyP). CyP was selected, as it has been used in the clinic to treat metastatic prostate cancer but it is only partially effective. Moreover, CyP has been demonstrated to have an immunosuppressive action, which has also been shown to potentially enhance the oncolytic ability of systemically administered G207 by increasing the replication and spread of virus within tumors. While prior experiments using intravenous administration of CyP have improved the effectiveness of HSV vectors for treating brain tumors, there are no reports on its effectiveness with viral vectors in prostate tumors. Therefore, subcutaneous TRAMP-C2 tumors were established in C57Bl/6 mice. Once established and growing, they were later treated with  $2 \times 10^7$  pfu of G207 via tail

**Fig. 3: Combination therapy of mouse prostate tumors with G207 and CyP.**

Subcutaneous TRAMP-C2 tumors established in C57Bl/6 mice were treated with  $2 \times 10^7$  pfu of G207 delivered intravenously by tail vein on days 1 and 4. One day prior to virus inoculation i.e., day 0 and 3, CyP was administered intraperitoneally at 25mg/kg.



vein on day 1 and 4 with intraperitoneal administration of CyP at 25 mg/kg one day prior to virus administration (days 0 and 3). As shown in **Fig. 3**, CyP+G207 was more effective in inhibiting TRAMP-C2 tumor growth as compared to either agent alone ( $p < 0.05$ ). While the combination of virus and CyP was clearly better than either CyP or G207 alone, it was interesting to note that these tumor cells responded rather markedly to even a low concentration (25mg/kg) of CyP alone. This above result was unexpected as published reports with human prostate cancer cell lines, such as LNCaP, DU145 etc, have shown resistance to CyP *in vivo*. Therefore, we tested the sensitivity of both human and mouse prostate cell lines to CyP *in vitro* and the results are shown below in **Fig. 4**.

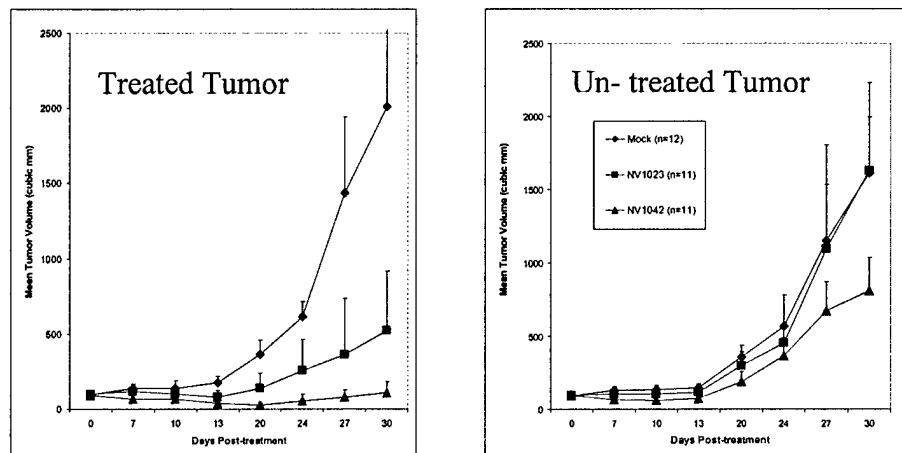


**Fig. 4: Sensitivity of Prostate Cell lines to CyP *in vitro*.** Prostate cancer cells were plated in triplicates and treated with varying concentrations of CyP 24 hours later. Two days post-treatment, cell viability was determined by Trypan Blue dye exclusion.

The *in vitro* data indicates that TRAMP-C2 tumors were the most sensitive cells to CyP as compared either to a second mouse prostate cell line, Pr14-2, or the human prostate cancer cell lines, LNCaP and DU145. Therefore, we are currently examining other chemotherapy agents, such as docetaxel and mitoxantrone in combination with G207 both *in vitro* and *in vivo*.

### **HYPOTHESIS 3. HSV vectors expressing soluble B7-1 and/or cytokines can stimulate systemic antitumor immunity effective against distant tumors.**

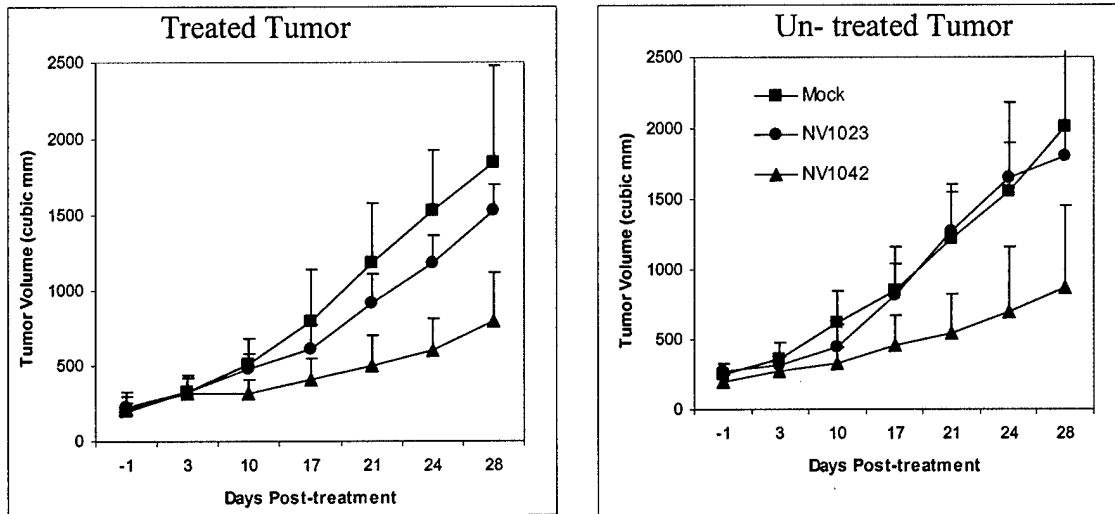
As stated in the previous progress report, our initial experiments comparing the G-series of HSV vectors, G207 and G47 $\Delta$ , with NV1023 from the N-series of HSV vectors, showed that NV1023 was the most effective virus against mouse prostate tumors. We also demonstrated that NV1042, derived from NV1023 with an additional insertion of the cytokine gene, mIL-12, was more efficacious in inhibiting tumor growth as compared to the parental vector, NV1023. Since NV1042 potentially can activate a more vigorous immune response against the tumors, it was important to demonstrate an immune effect on prostate tumors. Initial experiments with bilateral tumors, as described in our last progress report, demonstrated that in two different mouse prostate tumor models, TRAMP-C2 and Pr14-2, NV1042 was observed to be highly effective in the treated tumor and either marginally effective (TRAMP-C2) or not effective (Pr14-2) in the contralateral untreated tumors. In those studies, we had administered only 2 doses of NV1042. Therefore, with the goal of maximizing the immune effect, we repeated the bilateral tumor studies in both models with 4 intratumoral injections of  $1 \times 10^7$  pfu given unilaterally on days 0, 3, 7, and 10 into established, growing tumors. The results with TRAMP-C2 tumors shown in **Fig. 5** demonstrate that significant improved tumor regression occurred not only in the treated tumor with NV1042 ( $p < 0.005$  vs. mock;  $p < 0.05$  vs. NV1023) but also in the non-treated contralateral tumors with NV1042 vs. mock ( $p < 0.05$ ) and NV1042 vs. NV1023 ( $p < 0.05$ ).



**Fig. 5: Effect of unilateral intratumoral inoculation of NV1042 on contralateral un-treated TRAMP-C2 tumors.** Subcutaneous TRAMP-C2 tumors were established on bilateral flanks of C57Bl/6 mice and one of the flank tumor was treated intratumorally with either  $1 \times 10^7$  pfu of the virus (NV1023 or NV1042) or virus buffer (Mock) on days 0, 3, 7, and 10.

This immune effect on distant tumors was also observed with the second mouse prostate tumor model, Pr14-2, as shown in **Fig. 6**. Besides the direct cytolytic effect of the virus in

the injected tumors (NV1042 vs. NV1023:  $p < 0.05$ ), a marked tumor growth inhibition was observed (NV1042 vs. NV1023:  $p < 0.05$ ) in the non-injected tumor as well. Of note, the non-cytokine parent vector, NV1023 did not cause tumor regression in the contralateral tumors suggesting that the growth inhibition of the un-treated tumors was due to the expression of exogenous IL-12 delivered by the NV1042 virus.



NV1042 vs. Mock or NV1023:  $p < 0.05$  from day 10

NV1042 vs. Mock or NV1023:  $p < 0.05$  from day 21

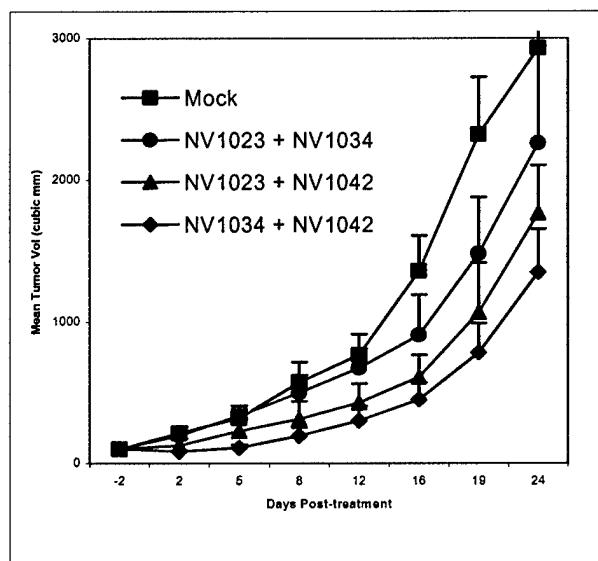
**Fig. 6: Effect of unilateral NV1042 on contralateral un-treated Pr14-2 tumors.**

Subcutaneous Pr14-2 tumors were established on either flanks of FVB/N C3(1) T Ag mice and one of the flank tumor was treated intratumorally with either  $1 \times 10^7$  pfu of the virus (NV1023 or NV1042) or virus buffer (Mock) on days 0,3,7, and 10.

Having established a potential immune effect on distant tumors with NV1042 administered intratumorally, it was important to explore its efficacy through systemic administration. As described in the prior report, our preliminary studies with intravenous NV1042 on TRAMP-C2 tumors showed significant tumor regression as compared to NV1023. Since with the contralateral tumor studies, we noted that multiple (more than two) administrations of the virus were required to demonstrate a significant effect in these mouse prostate tumor models, we chose to treat subcutaneous TRAMP-C2 tumors with intravenously delivered NV1042 on days 0,3, and 6. Additionally, we tested the combination of NV1042, an mL-12 expressing vector, and NV1034, a GM-CSF expressing vector (also derived from NV1023), in this model. GM-CSF is a cytokine involved in initiating an immune response by activating antigen presenting cells, where as IL-12 acts at the effector phase of the immune response in activating and recruiting T cells. Thus, we reasoned that a combination of the two cytokine containing viruses, NV1034 and NV1042 would enlist a more robust anti-tumor immune response. Results, as shown in Fig. 7 indicate that the combination of NV1042 and NV1034 was significantly more effective than any of the other combinations tested ( $p < 0.05$ ).

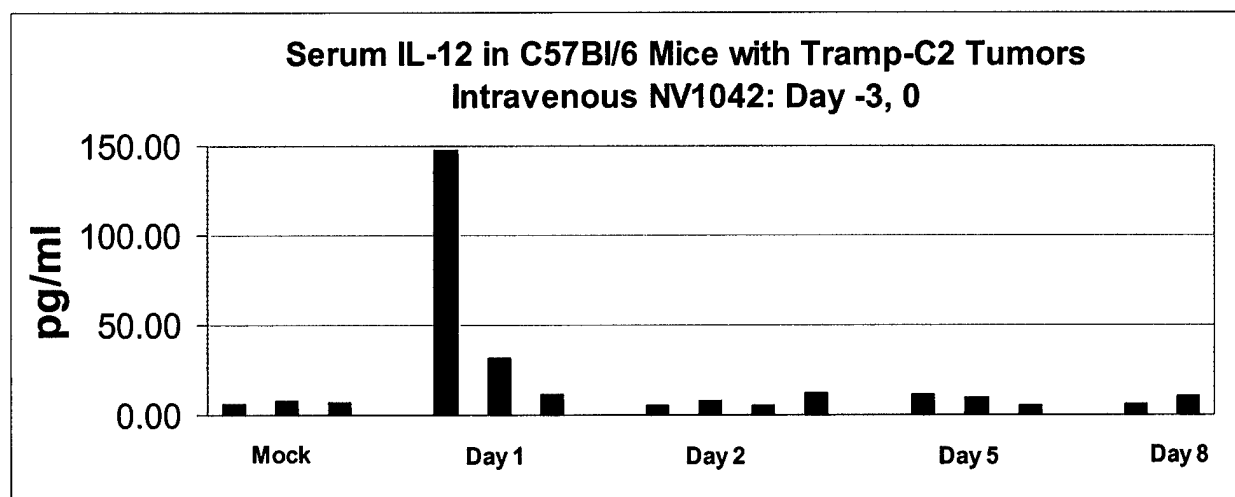
In order to assess the extent of infection and replication of the systemically administered NV1042 virus within tumors and to evaluate whether IL-12 expressed by NV1042 was exerting a systemic effect or a local effect, we treated subcutaneous tumor bearing mice with intravenous (tail vein) NV1042 on day -3, and 0. Groups of mice were sacrificed on





**Fig. 7: Efficacy of the combination of an IL-12 vector, NV1042 with a GM-CSF vector, NV1034, on the growth of mouse prostate tumors.** Subcutaneous TRAMP-C2 tumors established in C57Bl/6 mice were treated intravenously (tail vein) with a combination of virus at  $1 \times 10^7$  pfu of each virus on days 0, 3, and 6.

day 1, 2, 5, and 8. Blood was collected for determining the levels of serum IL-12 and the results are shown in **Fig. 8**. Results show that there is only a transient systemic increase of IL-12 levels in the serum on day 1 post-virus inoculation, and by day 2 and thereafter the serum IL-12 levels are at the basal levels similar to the mock-treated animals. The tumor and major organs were also collected for bio-distribution analysis and are currently being evaluated by X-gal staining for lacZ expression from virus and PCR analysis of viral sequences.



**Fig. 8: Serum IL-12 levels in TRAMP-C2 tumor bearing C57Bl/6 mice.** Subcutaneous TRAMP-C2 tumor bearing mice were treated intravenously with NV1042 on days -3, and 0. Each bar represents one mouse. IL-12 measurements were done by ELISA assay.

#### PLANS FOR THE UPCOMING YEAR:

In this next year we will perform:

**HYPOTHESIS 1:** Having shown that HSV can efficiently infect and kill dividing endothelial cells *in vitro*, we will now study the effect on tumor vasculature *in vivo*

HYPOTHESIS 2: Having demonstrated the beneficial interaction of cyclophosphamide and G207, we will study other chemotherapy agents commonly used to treat prostate cancer, such as docetaxel and mitoxantrone in combination with G207 both *in vitro* and *in vivo*.

HYPOTHESIS 3: Having demonstrated the beneficial immune effects of one or two cytokine releasing vectors on distant tumors, we will now study this in a metastatic prostate cancer model.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Dextran sulfate was effective in enhancing the tumor inhibitory activity of G207.
- G207 can efficiently infect human endothelial cells (HUVEC) even at a very low MOI.
- The combination of G207 and cyclophosphamide was clearly better than either alone.
- In two different model systems, improved tumor regression occurs with cytokine releasing vectors not only in the treated tumor but also in non-treated contralateral tumors.
- The combination of two cytokines was more effective than one.

**REPORTABLE OUTCOMES:**

We are now using this data to prepare:

- An R01 grant to the NIH
- We expect several manuscripts to result from the above data

**CONCLUSIONS:** This work demonstrates that the basic concept of local therapy using oncolytic vectors can be extended for use as systemic therapy for metastatic prostate cancer employing several different complementary mechanisms.

**REFERENCES:** none necessary

**APPENDICES:** none